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14. ABSTRACT

This work describes studies into the mechanism of action of bisperoxovanadium (bpVs) compounds and into the basic biology and regulation of Cdc25A phosphatase. The bpVs cause phase-specific cell cycle arrest (G1/S), dose-dependent inhibition of Cdk activity, and persistent Rb hypophosphorylation upon release from serum starvation, consistent with Cdc25A inhibition. Further, they cause p53-independent apoptosis. Oxidative stress and DNA damage do not appear to be involved in their mechanism of action, given that: p53 is not induced by bpV(Me2Phen); p53 and p21 status do not affect IC50; depletion of glutathione or supplementation with antioxidants does not affect IC50, in contrast to other heavy metal-based agents. With respect to regulation, the Cdc25A nuclear localization signal (NLS) was identified and characterized. Data suggest that phosphorylation of S292, adjacent to the NLS, may promote nuclear localization. In the unperturbed cell cycle, S292 phosphorylation, a Chk1/2 target, appears to label sites of local inhibition of Cdc25A, suggesting fine tuning of the Cdc25-Cdk axis at the scale of specific subnuclear and mitotic structures.

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Introduction

This funding was provided to support the study of bisperoxovanadium compounds as potential Cdc25A inhibitors. Cdc25A is considered a rational target for chemotherapeutic drug development given its role as a key regulator of cell cycle progression, its ability to transform rodent fibroblasts, and its observed overexpression in several malignancies. Bisperoxovanadium compounds have received a certain amount of attention from the research community as stable vanadium conjugates showing activity as phosphatase inhibitors and as insulin mimetics. Among the key research goals of this award were mechanistic studies into bpVs to ascertain whether or not they induce oxidative stress or DNA damage as alternative explanations for the observed cell cycle arrest. We were able to demonstrate, by use of agents that deplete or supplement intracellular antioxidant stores, that cell killing by bpVs, unlike that of simple vanadium salts, is independent of oxidative stress. Further, using several measures such as induction of DNA damage response proteins and a sensitive assay for DNA strand breaks (the comet assay), we provided evidence that bpVs do not induce DNA damage under the tested conditions. We have shown that bpVs have significant *in vivo* activity, reducing the rate of tumour growth by some 80% in two syngeneic murine models. In concert with these studies, we made several observations regarding the basic biology of Cdc25A. From the literature, we noted that several reports suggested cytoplasmic localization of Cdc25A, contrary to the consensus nuclear localization generally described. Further, control of localization of Cdc25s by kinases such as Plk1 and Chk1 is considered pivotal to the regulation of cell cycle timing and arrest. We reasoned that the regulation of Cdc25A might be regulated in a similar manner, and indeed, that Cdc25A's cell cycle functions may be restricted to the nucleus, while cytoplasmic Cdc25A may have an as-yet unrecognized function independent of mitosis. We observed that a Cdc25A harbored a sequence highly similar to a consensus bipartite nuclear localization signal. Further, flanking phosphoacceptor residues show a remarkable conservation, including S292, which matches a consensus PKA phosphorylation site. Through a number of techniques, including site-directed mutagenesis, GFP-

tagging, and immunofluorescence with phospho-specific antibodies, we provided data indicating that S292 phosphorylation may promote nuclear localization of Cdc25A, but that it is associated with inactive Cdc25A in the local context. These data suggest that Cdc25A is subject to local fine-tuning in a cell cycle-dependent fashion. Importantly, these data indicate that dysregulation of Cdc25A activity may be accomplished through alternative avenues such as Chk1 or ROCK inhibition; such strategies may be required given that the peculiarities of the Cdc25A catalytic site have been seen as barriers to the generation of effective inhibitors of this enzyme.

Body

This work sought to better understand the mechanism of action of bisperoxovanadium compounds as well as to test the principle of Cdc25A inhibition as a strategy for chemotherapeutic drug development.

NaOV and simple peroxovanadium species can be cytotoxic; it is also notable that some species have prominent effects on cell cycle progression. For instance, it was noted that the addition of either of two bpVs, bpV(Phen) or bpV(Pic) to cell culture medium resulted in G2/M arrest (1). The inhibition of cell cycle progression is an interesting observation in light of the fact that the serine/threonine phosphatase inhibitors microcystin and OA have the opposite effect; that is, they act as tumor promoters, and particularly given the fact that certain vanadium and peroxovanadium species appear to promote RTK signaling, a phenomenon one would expect to lead to enhanced proliferation. The fact that some peroxovanadium compounds cause cell cycle arrest implies the inhibition of an “activating phosphatase”. That is, certain phosphatases, including most Ser/Thr phosphatases and receptor-associated tyrosine phosphatases function to shut off intracellular signaling pathways, and therefore dampen mitogenic signaling. Activating phosphatases, in contrast, remove inhibitory phosphorylations and promote cell cycle progression. The Cdc25 family are among the rare activating phosphatases, and therefore represent prime suspects for the cell cycle inhibitory properties of peroxovanadium species. Another “activating phosphatase” is the T-cell Protein Tyrosine Phosphatase TCPTP (A.K.A. PTP-S2), which can increase proliferation while shortening G1 (2).

The cytotoxic effects of some vanadium compounds led to the suggestion that they might be useful as antineoplastics. Several vanadium species (orthovanadate, vanadyl sulphate, and peroxovanadate were observed to inhibit growth of a number of cell lines *in vitro*, with IC_{50}s in the low micromolar range (3). Interestingly, the addition of H_2O_2 was found to potentiate the cytotoxicity of orthovanadate, suggesting that either peroxovanadate species thus formed are more active or that oxidative stress plays a role in cell killing, and that the two compounds synergize in producing ROS.

Finally, a single 500 μg injection of orthovanadate was found to reduce tumour weight in an murine MDAY-D2 model (3).

Our studies, reported in *Molecular Cancer Therapeutics* (4), made the following key observations:

Inhibition of Cdc25A in vitro. Using purified GST-fusion proteins (Cdc25A and hVH2) and commercially available tyrosine phosphatases (YOPH, TCPTP, and LAR), a small panel of bpVs and the salt NaOV was tested for the ability to inhibit Cdc25A in vitro. Cdc25A was observed to be the most or second-most (behind YOPH) inhibited of the tyrosine and dual-specificity phosphatases tested. Moreover, it evident from these studies that the organic heteroligand of the bpVs modulated the potency of the agents towards a given phosphatase, suggesting that it may indeed be possible to increase specificity towards a phosphatase through the judicious choice of heteroligand.

Inhibition of Cdc25A in whole cells. Cell cycle analyses demonstrated that treatment of unsynchronized cells with concentrations of bpV(Me2Phen) just superior to their 96 hour IC_{50}s resulted in arrest in G1 or early S phase, while treatments very near their 96 hour IC50s resulted in a broad arrest throughout S phase. These data are in agreement with the reported role for Cdc25A in regulating progression through G1/S. More directly, immunocomplex kinase assays demonstrated a dose-dependent inhibition of Cdk2 upon treatment with the bpVs, consistent with inhibition of Cdc25A.

Cytotoxicity of bpV compounds. More than two dozen cancer cell lines were tested for bpV(Me2Phen) and bpV(Phen) cytotoxicity, with all showing IC_{50}s in the sub- to low micromolar range. Further, it was demonstrated by DNA laddering that bpV(Phen) causes apoptosis.

Antineoplastic activity in murine models. Repeated IP injections of bpV were shown to reduce tumour growth in syngeneic mouse models by up to 80%. Two models were used, Lewis Lung carcinoma in Bl/6 and DA3 in Balb/c mice. In both models, IP injections were associated with acute (lethargy) and chronic (dehydration, weight loss, diarrhea) toxicities. We hypothesized that some of these toxicities might be due to the insulin mimetic properties of bpVs, and attempted to mitigate them by adding dextrose to the injection vehicle, but we observed no improvement in this regard.

Mechanism of action. bpV(Me2Phen) treatment of p53 wild-type MCF-7 did not induce p53 stabilization, suggesting that the observed loss of Cdk activity and cell cycle arrest were not due to induction of a cell cycle checkpoint. Further support for this notion comes from the observations that p53 or p21 knockout HCT116 cells do not substantially differ from their wild-type counterparts in their susceptibility to bpV-mediated cell killing. Further, treatment MDA-MB431 cells with bpV(Me2Phen) did not appear to cause a mobility shift (indicative of activation) of Chk1, though Chk1 levels were observed to decline at later timepoints; the cause of this decline in Chk1 levels is not currently known. A comet assay was used to assay DNA damage more directly. The comet assay (or “single cell gel electrophoresis”) measures the generation of sub-chromosomal DNA fragments by treatment with agents which directly break DNA or which form adducts which are substrates for DNA repair enzymes. We observed that bpV treatment alone did not generate fragments at a rate higher than background, though there was a synergistic generation of DNA damage upon treatment with bpV(Me2Phen) and UV light. This contrasted with the additive level of damage upon treatment with As2O3 and UV light. bpVs have been reported to cleave naked (plasmid) DNA upon UV treatment (photolysis). This result suggests that, while bpV(Me2Phen) does not appear to be generating damage directly, it may be in close proximity to the chromatin.

Oxidative stress. Other groups have suggested that oxidative stress may play a role in the cytotoxicity of related bpVs (5). We therefore tested whether a similar process was at play in the case of bpV(Me2Phen) by depletion of the intracellular glutathione with the γ-GCS inhibitor BSO. In the complementary assay, we supplemented the growth medium with the enzymatic antioxidant superoxide dismutase, or with cysteine or glutathione. In none of the above circumstances did modulation of antioxidant levels affect sensitivity to bpV(Me2Phen), though dramatic sensitization to NaOV, As2O3, and Sb2O3 were observed upon BSO treatment. These data suggest that oxidative stress does not substantially contribute to the cytotoxicity of bpV(Me2Phen).

In addition to the above positive results, certain observations argue against the specificity of bpV(Phen) and bpV(Me2Phen). Most importantly, the levels of tyrosine phosphorylation of multiple proteins increase upon bpV(Phen) treatment, strongly suggesting that

multiple phosphatases are inhibited by this compound. It is also evident from our in vitro inhibition studies that the entire panel of phosphatases tested was inhibited to some extent by the bpVs. Further, as noted above, bpV(Phen) and other compounds have been shown to have insulin mimetic properties, and this associated with prolonged activation of the insulin receptor believed to be caused by inhibition of a tyrosine phosphatase. Finally, the results of our in vivo studies indicated significant toxicities associated with the use of bpV(Me2Phen). These were comparable to those previously reported for vanadium compounds. The presence of side effects is often taken to be an indication of “off target” effects, though the possibility that they represent “on target” effects (i.e. unexpected effects resulting from inhibition of Cdc25A itself) cannot be excluded.

None of these observations precludes the further development of bpVs as potential chemotherapeutic agents. Significantly, our observations demonstrate significant modulation of potency and specificity by the organic heteroligand, suggesting that further substitutions may result in inhibitors with enhanced specificity for Cdc25A. Nonetheless, several researchers have attempted to find viable Cdc25A inhibitors, with generally poor results. The difficulty has been ascribed to the nature of the Cdc25A catalytic site (6). In this context, we considered that a better understanding of the basic biology of Cdc25A might reveal novel means by which to inhibit the enzyme. Specifically, regulation of Cdc25C by localization has been shown to be key in regulating normal cell cycle progression and the response to DNA damage (7;8). We therefore examined the nuclear localization of Cdc25A in order to determine whether this, too, was a regulated process.

With respect to this axis of the research, the following observations were made:

Cdc25A localization determinants. Cdc25A was found to harbour a classical bipartite nuclear localization signal (NLS). Mutation of a portion of the NLS by site-directed mutagenesis was found to disrupt nuclear localization of the enzyme, demonstrating that it is indeed necessary for nuclear localization. Further, subcloning of this region and fusion to GFP demonstrated that it is the wild-type, but not mutant, sequence is sufficient for nuclear localization. These results were later confirmed by another research group (9). The

Cdc25A NLS is characterized by well-conserved flanking phosphoacceptor sites, raising the possibility that the observed nuclear localization might be regulated by their phosphorylation. Indeed, the most highly conserved of these sites, S292, conforms to a consensus PKA/CamKII phosphorylation site. Using site-directed mutagenesis and in vitro kinase assays with these two kinases, We demonstrated that S292 is indeed the sole site of phosphorylation by PKA in vitro, while CamKII either does not phosphorylate S292 or phosphorylates it only as one of several sites within Cdc25A. We was unable, however, to conclusively demonstrate an in vivo association between PKA and Cdc25A. At roughly this time, another group published an article showing that S292 is phosphorylated in vivo, and that both Chk1 and Chk2 could phosphorylate this site in whole cells. We sought to examine the in vivo consequences of S292 phosphorylation, and hypothesized that S292 phosphorylation might cause nuclear exclusion by ablating the NLS. Contrary to this hypothesis, we observed that upon transfection of Cos-7 cells with GFP-fusions of a phosphomimetic mutant (S292E), increased nuclear localization relative to a WT or catalytic mutant (C430S) GFP fusion was apparent. This suggests that phosphorylation of S292 may, in some contexts, promote nuclear localization.

Generation of a phospho-specific antibody for pS292. In order to gain a better understanding of how S292 phosphorylation may be regulated in a physiological context, we generated a rabbit polyclonal serum against pS292. ELISA testing demonstrated several-fold preference for the phosphorylated versus non-phosphorylated epitope. We made use of the capacity of PKA to phosphorylate S292 to demonstrate the capacity of this antibody to detect phosphorylated GST fusion proteins in western blot. We further demonstrated the ability of this serum to detect ectopic Cdc25A, but not S292 mutants, when overexpressed in U2OS or EKVX cells, and showed a strong correlation between the intensity of staining with a commercial Cdc25A antibody and the pS292 serum in immunofluorescence.

Immunofluorescence with pS292 antibody. Immunofluorescence with the pS292 antibody indeed demonstrated predominantly nuclear staining, in accord with the GFP-fusion studies described above and with the reported localization of Cdc25A in the literature. There was, however, a discrete pattern of localization to subnuclear

compartments that has not previously been reported. Structures labelled included condensed chromatin, puncta on metaphase chromosomes (possibly reprepresenting kinetochores), the actomyosin contractile ring (during anaphase), and the midbody. Further, discrete nuclear bodies were labelled during interphase.

Mass spectrometry. The antigenic peptide was used as bait for mass spectrometry in order to better understand the nature of this localization. Interactions fell into three major categories: chromatin remodelling/replication proteins (e.g. SMARCA), cytoskeletal proteins (e.g. α-actinin, non-muscle myosin), and vesicle trafficking proteins (e.g. Snx6, Snx27).

Interpretation: Nuclear bodies. Cdc25s are master regulators of the cell cycle and cytokinesis, which is, at some levels, a very physical process. Cdc25A is thought to regulate Cdk2 activity by dephosphorylating Y15. A paradoxical observation is that Cdk2 phosphorylation at this site increases as cells enter S phase, the phase of maximal Cdk2 activity (10). An explanation for this finding is presented by the results of Shechter et al. (11), who demonstrated that the generation of ssDNA during normal replication of Xenopus cells causes activation of ATR and ATM. This local activation is thought to repress the firing of adjacent origins of replication. The downstream targets of ATR and ATM are Chk1 and Chk2. Recall that S292 has previously been reported to be a Chk1/Chk2 target (12). The pS292 nuclear bodies observed in interphase cells may thus represent repressed origins of replication. Consistent with this hypothesis, the staining of these bodies is diminished by actinomycin D, which halts DNA replication at the primase step (i.e. prior to generation of ssDNA by firing of the replicative helicase), but not by aphidicolin treatment (which halts DNA replication by poisoning the polymerase, leading to generation of ssDNA). Further, crude extract kinase assays indicate an increase in kinase activity towards S292 as cells entered S phase.

Interpretation: mitotic structures. The timing of Cdk1 activation and inactivation is key to cytokinesis. Two model organisms differ in their strategies for inactivating Cdk1. In S. cerevisiae, Cdk1 is inactivated at metaphase exit by cyclin B degradation (as is generally thought to be true of higher eukaryotes); in S. pombe, Cdk1 is inactivated by the Cdc14-dependent

dephosphorylation of Cdc25 followed by its degradation. It has recently been observed that mammalian Cdc25A is similarly a Cdc14 target and that it is degraded at metaphase exit. Further, it has been reported that Cdk1 activity begins to drop prior to Cyclin B1 degradation, suggesting that post-translational mechanisms, rather than simply degradation, regulate its activity at metaphase exit. We propose, therefore, that pS292 represents sites of local Cdc25A inactivation, allowing a fine-tuning of Cdk1 activity, such that it may be inhibited by concerted Wee1 activity and Cdc25A inhibition at the local level despite persistent high levels of activity in the cell. Several precedents for such a notion exist. The centrosomal pool of Cdk1 is activated prior to the rest of the cellular Cdk1 complement, demonstrating the possibility of (Cdc25-dependent) local fine-tuning. Further, Plk1, like Cdk1 and Cdc25A, is degraded by the APC at metaphase exit. However, a small pool of Plk1 exists at the midbody, and this pool represents highly active kinase (Francis Barr, personal communication). Additional support for this notion arises from the observation that ROCK, a known modulator of actomyosin contractility, has been shown to inhibit Cdc25A (13). The precipitation of several components of the actomyosin ring complex by the antigenic peptide and the immunofluorescence labeling of this structure by the pS292 antibody are consistent with the regulation of actomyosin contractility by Cdc25A activity.

Key research accomplishments

In this research we provided strong evidence that bpVs inhibit Cdc25A in vitro an in vivo. Further, we demonstrated in vivo antineoplastic activity in murine models, albeit with associated toxicity. We provided evidence that oxidative stress and DNA damage are unlikely to contribute to the mechanism of bpV-mediated cell killing. We subsequently conducted studies into the basic biology of Cdc25A, identifying and characterizing its nuclear localization sequence. We provided evidence of phosphorylation-mediated regulation of Cdc25A nuclear localization. We subsequently generated and characterized a phospho-specific antibody to this epitope and demonstrated the labeling of numerous nuclear bodies and mitotic structures. We provided evidence through mass spectrometry, cell cycle analysis, and immunofluorescence that these sites may represent sites of local regulation of the Cdc25A/Cdk signaling module, a notion supported by the literature.

Reportable outcomes

- One (1) Manuscript
  - **Scrivens P.J.**, Alaoui-Jamali M.A., Giannini G., Wang T., Loignon M., Batist G., Sandor V.A. *Cdc25A-inhibitory properties and antineoplastic activity of bisperoxovanadium analogues.* Molecular Cancer Therapeutics 2003 Oct;2(10):1053-9.
- Two (2) Meeting abstracts
  - **Scrivens, P.J., Alaoui-Jamali, M.A.** *Regulation and chemotherapeutic targeting of Cdc25A.* FD Era of hope June 8-11, 2005, Philadelphia, PA. (Poster)
  - **Scrivens, P.J.**, Alaoui-Jamali, M.A. *Phosphorylation-dependent Localization of Proteins to the Midbody of Human Cells*. AACR: Cell Cycle and Cancer: Pathways and Therapies. December 1-5 2004, Fort Lauderdale, Fla. (Poster)
- One Ph.D. Thesis
  - **Scrivens, P.J.**, *Regulation and Chemotherapeutic Targeting of Human Cdc25A Phosphatase*, (successfully defended July 26, 2007).

Conclusions

This research project accomplished many of the goals in the original SOW, providing valuable insights into the mechanism of action of a potential novel class of chemotherapeutic agents, the bisperoxovanadium compounds. Further, important advances were contributed to our knowledge of the basic biology and regulation of Cdc25A. We have provided evidence of phosphorylation-mediated regulation of Cdc25A nuclear localization, and of local fine-tuning of Cdc25A activity during unperturbed transit through S and M phases. Given that Chk1, and possibly Chk2 and ROCK, are important regulators of Cdc25A at this site, these findings lend support to the development of Chk1 inhibitors to disrupt the normal function of the Cdc25A-Cdk axis in proliferating cells. It is also important to note that Cdc25A has been reported to be cytoplasmic in several tissues such as neurons and regenerating liver after partial hepatectomy. The presence of Cdc25A in some non-proliferating tissues such as neurons and heart muscle may suggest functions of Cdc25A independent of proliferation. The localization of Cdc25C to the nucleus has been seen as critical its their function as an inducer of mitosis (7;8). It may thus be reasonable to attempt inhibition of Cdc25A nuclear localization as a strategy to specifically block the proliferative functions of this phosphatase while sparing cytoplasmic (and presumably non-proliferative) functions.

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Appendices

None